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A NATURAL SUBSTANCE THAT REGULATES THE CELL CYCLE IN COMPLEX PLANT TISSUES

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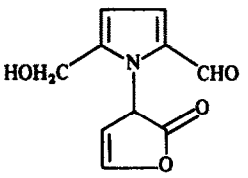
Key Word Index—*Pisum sativum*; Leguminosae; trigonelline; cell cycle; 1-(3-(4,5-dihydro-2-furanone)-5-hydroxymethyl)pyrrole-2-carboxyaldehyde.

Abstract—A natural substance which regulates the cell cycle of seedling roots of *Pisum sativum* has been isolated and identified as 1-(3-(4,5-dihydro-2-furanone)-5-(hydroxymethyl)pyrrole-2-carboxyaldehyde. This compound interacts with trigonelline to determine the percentages of cells in G1 and in G2 in pea root meristems. Both purified natural and synthetic compounds are active at concentrations of 5×10^{-6} M.

INTRODUCTION

The search for natural substances that regulate the cell proliferation cycle in complex tissues began with the investigations of Bullough and co-workers in the early 1960's [1, 2]. Since these initial investigations several studies of these substances have focused on chalones, which regulate cell proliferation mammals [3]. Understanding the chemical structures and physiological activities of such substances is critical to understanding how cell division is controlled as well as how these natural controls may be modified in cancer.

Recently, trigonelline (*N*-methylnicotinic acid) was shown to arrest cells of both roots and shoots of legumes in G2 during natural cell differentiation [4, 5]. Much information has been accumulated about the metabolism of this hormone [6, 7]. It appears that trigonelline influences cells in the cortex to arrest in G2 [8]. Such cells appear to be the preferential targets of *Rhizobium* infection for nitrogen fixation in legumes [9, 10]. Recently, the chemical identification of 1-(3-(4,5-dihydro-2-furanone)-5-hydroxymethyl) pyrrole-2-carboxyaldehyde **1** from pea tissues was made [11]. The present investigations document that **1** interacts with trigonelline to regulate the cell cycle in seedling roots of garden peas.



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RESULTS

Experimental results (Table 1) show that a substance antagonistic to trigonelline's action is present in cotyledons of 10-day-old seedlings relative to cotyledons of three-day-old seedlings. Specifically, results in Table 1 show that cotyledons from three-day-old seedlings provided 40% cells arrested in G2 in stationary phase meristems when roots from three-day-old seedlings were examined. However, when cotyledons of older seedlings were cultured with roots from three-day-old seedlings, lower percentages of cells arrested in G2 occurred. These resultesults are similar to those shown previously [12]. The differences in the percentages of cells in G2 in roots of three-day-old seedlings were not related to trigonelline concentrations in the medium. Specifically, trigonelline concentrations in culture media were all similar (Table 1) and all were above the 10^{-6} M trigonelline threshold concentration needed for effect [8].

Table 1. Concentrations of trigonelline in 50 ml distilled water exposed to 20 cotyledons from seedlings with various ages incubated under aseptic culture conditions and proportions of cells in G2 in stationary phase of *Pisum sativum* after exposure to medium with cotyledons of various aged seedlings

Age of the seedlings for which cotyledons were used (days)	Trigonelline Concentration (M) of the distilled water in which cotyledons were incubated	Percentages of cells arrested in G2 in stationary phase meristems*
3	1.26×10^{-5}	40
5	1.26×10^{-5}	32
7	9.4×10^{-6}	21
10	9.2×10^{-6}	21

*The standard error of the mean of all samples was 2%. These values are similar to values obtained in previous experiments [5].

Purified **1** from hypocotyls and cotyledons of 10-day-old seedlings and synthetic **1** were antagonistic to trigonelline in culture with roots of three-day-old seedlings (Table 2). When trigonelline was added at a concentration of 10^{-6} M, the percentage of cells in G2 was usually

Table 2. Antagonistic effect of a HPLC fraction which contained 1, natural or synthetic, on promotion of cell arrest in G2 by trigonelline

Experiment-treatment	Mean percentage of cells arrested in G2*
HPLC fraction	
No additions to media	16
Trigonelline (10^{-6} M) added	38
Trigonelline (10^{-6} M) added with an HPLC fraction of a plant extract	11
Natural 1	
No additions to media	25
Trigonelline (10^{-6} M) added	43
Trigonelline (10^{-6} M) added with natural 1	16
Synthetic 1	
No additions to media	13
Trigonelline (10^{-6} M) added	30
Trigonelline (10^{-6} M) added with synthetic 1	17

*The standard error of the mean of all samples is 2%.

between 30 and 43%, similar to previous results [5]. However, when partially purified plant extracts, or purified 1 from 10-day-old seedlings or synthetic 1 were added to culture medium with trigonelline, the percentages of cells in G2 were similar to controls (no additives to medium). These results suggest that 1, at a concentration of 5×10^{-6} M, has an antagonistic effect on trigonelline's promotion of cell arrest in G2 in *P. sativum*.

DISCUSSION

Previous research has centered upon the physiological response of cells to trigonelline [8, 11], the specificity by which the trigonelline molecule controls cell arrest in legume roots [13], and the metabolism of trigonelline within the pyridine nucleotide pathway for the synthesis of NAD [6–8]. The source of trigonelline in young seedlings is the cotyledons. Trigonelline is transported from cotyledons to roots and shoots where it promotes preferential cell arrest in the G2 phase of the cell cycle. As seedlings age from three to 10 days of age, the trigonelline concentration in root meristems decreases from 90 to 30 μg/g tissue. Concomitant with the decrease in trigonelline concentrations there is a decrease in the proportion of cells arrested in G2 from 60 to 20%. In previous experiments in which the proportion of cells arrested in G2 decreased from 40 to 20% in roots of three-day-old plants incubated with three- and 10-day-old cotyledons, it was thought that the decrease in the percentage of cells in G2 was due to a decrease in trigonelline in solution from the cotyledons. The experimental results presented here demonstrated that the decreased percentage of cells arrested in G2 was not due to a change in trigonelline concentrations. This finding triggered the search for the antagonistic compound [11].

Roots of 10-day-old plants were selected as the preferred tissue to recover the antagonistic compound 1 since the mass of roots was much higher than the mass of cotyledons and if the compound is truly an antagonist to trigonelline it should be present in roots. Extracts were continually tested in the bioassay. The extraction and purification procedures provided about 20 μg of pure antagonist for each 500 g of roots fresh weight [11]. It would appear that the relatively high proportion of root cells arrested in G2 in three-day-old seedlings may be a function of relatively high concentrations of trigonelline concomitant with low concentrations of this antagonistic substance 1. In contrast, the lower percentage of cells arrested in G2 in 10-day-old seedlings may arise from lower trigonelline concentrations concomitant with high concentrations of 1.

EXPERIMENTAL

The methods to extract, purify, and chemically identify 1 have been described previously [11]. In addition, the synthesis of 1 has been described in detail [11]. Pure 1 was tested for biological effectiveness in a standard bioassay.

Biological activity of 1 and plant extracts was determined using a root bioassay as previously described [8]. Over a period of several years many plant extracts were placed in this bioassay to test for antagonism to trigonelline's function. In the bioassay, seeds of *Pisum sativum* were surface-sterilized and germinated in sterile vermiculite. Under sterile conditions excised root tips from 3-day-old seedlings were placed in medium with sucrose with the presence or absence of trigonelline and the presence or absence of root extracts, the isolated antagonistic substance or synthetic antagonistic substance. After 3 days of culture, root meristems were transferred to carbohydrate-free medium for 4 days, so that all cells were in the G1 or G2 stages of the cell cycle. The percentages of cells in G1 and in G2 in these carbohydrate-starved meristems is highly correlated with the percentages of cells present in mature root tissues under natural growing conditions [14].

To determine the percentages of cells in G1 and in G2, roots were fixed in EtOH–HOAc (3:1). Relative amounts of DNA per nucleus were determined on Feulgen-stained nuclei from 0 to 2 mm terminal meristems by microfluorimetry [15]. To determine the amount of trigonelline in solutions, cotyledons of various aged seedlings were incubated in distilled water or nutrient medium for three days. Aliquots of these solutions were spotted on silica gel TLC UV plates, 250 μm thick (Analtech). Extracts were developed in Me_2CO–H_2O (1:1). Plates were allowed to dry in air and trigonelline was eluted. Quantities of trigonelline were determined by HPLC [16] using a Whatman Partisil-Sax 10 column using 7 mM K-Pi as solvent (pH 5.8) at a flow rate of 1 ml/min. Recovery of trigonelline from TLC plates was usually *ca* 50%. For each experiment, authentic trigonelline (Sigma) was applied to the TLC plates, eluted and processed like plant extracts through HPLC. Plant samples were corrected by the recovery percentage for each experiment.

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